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Antibiotic Substances Produced by Soil Bacteria
J. C. HOOGERHEIDE 599

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H. A. GLEASON AND E. H. FULLING
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by
E. H. FULLING

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ANTIBIOTIC SUBSTANCES PRODUCED BY SOIL BACTERIA

J. C. HOOGERHEIDE
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INTRODUCTION

The name "antibiotic substances" refers to a group of substances of divergent chemical nature which are produced by certain species of micro-organisms and which have definite bactericidal or bacteriostatic properties against other groups of micro-organisms.

The enormous popularity that this group is enjoying at present is largely due to the fact that certain members of it, especially penicillin, not only are potent bactericidal agents but also possess remarkable therapeutic potentialities. The feverish research being conducted in this newly discovered field is at present directed mainly toward the discovery of new chemotherapeutic agents, and in many instances is approaching the dimensions of a scientific gold-rush.

Although the possibilities for discovery of such antibiotic substances are limitless, it is by no means certain that this field will prove to be the rich mine for therapeutic agents that many investigators believe it to be. Currently there are no scientific facts to justify such expectations. The mere fact that bactericidal substances are excreted by living organisms unfortunately does not necessitate that such agents have therapeutic value or would be less toxic for humans than bactericidal chemicals, such as phenols, mercurials or certain detergents. On the other hand, the mere fact that the organism which excretes such substances apparently is not affected by them, indicates that there is a marked specificity for destruction of certain living cells, whereas other living cells are not affected. Such a high degree of specificity is rarely encountered among the common bactericidal chemicals and, as is the case with penicillin, opens the possibility that certain antibiotic agents may exhibit specificity for certain disease-producing bacteria and be relatively non-toxic to human cells.

Although the existence of antibiotic substances has been known since the early days of bacteriology, it was not until recently that the field was explored. Undoubtedly the stimulus for this new exploration was the sudden revival of interest in chemotherapy, growing from the discovery of sulfa drugs. Sulfanilamide was known to the chemists for a long time, but it was not until about thirty years after its discovery that its therapeutic potentialities were recognized. This discovery at once revived the almost dormant science of chemotherapy and gave the research worker in this field renewed confidence in the belief that infectious diseases could be cured by the use of chemicals.

The bacteriologist, by the nature of his profession, was among the first to realize the major importance of the discovery of sulfa drugs, and the short step from the field of sulfa drugs to that of antibiotics was a logical development.

In the early days of bacteriology, more than sixty years ago, it was observed repeatedly that when several bacterial species were allowed to grow together on the same nutrient plate, a clear and sterile halo sometimes developed around the colonies of certain species. Apparently the bacteria of such a colony excreted a substance into the medium which prevented bacteria of non-related species from growing close to such a colony. This phenomenon, referred to in the older literature as "bacterial antagonism", was studied quite extensively at the turn of the century. A large number of such "antagonistic" strains was isolated. Each of them specifically inhibited the growth of one or more species under certain conditions. A considerable strain-specificity was observed among these antagonists, and usually only a few strains of the same species possessed such antagonistic properties.[1]

Already early investigations revealed that the phenomenon of bacterial antagonism, in general, could not be explained by the assumption that the medium was exhausted by the growth of the antagonist, nor could it be explained by the excretion of the usual metabolic products of the antagonists, such as organic acids, alcohol, ammonia or indole. Soon it was realized that certain strains under specific conditions of growth are able to excrete into the medium, substances of high bactericidal potency for other species. Garré (2) pointed out as early as 1887 that the presence in the soil of such

[1] For an excellent review on bacterial antagonism see: 145.

bactericidal substances, excreted by certain types of soil bacteria, may be the explanation for the rapid destruction of pathogenic organisms when they are added to the soil. The same investigator expressed the opinion that eventually such bactericidal agents, excreted by soil bacteria (antibiotics), might be used as valuable therapeutic agents for treatment of bacterial diseases.

Although considerable work was done in this direction, little progress was made. Attempts to isolate and purify the bactericidal products usually met with little success, and finally the idea was entirely dropped as being impractical. Pyocyanase, a thermostable agent of lipoid nature, excreted by certain strains of *Pseudomonas aeruginosa,* was probably the first antibiotic substance isolated at that time with any degree of purity. Not until five years ago, however, was the study of bacterial antagonism renewed, and special emphasis was then laid on the isolation and purification of antibiotic substances from culture media.

TYROTHRICIN (GRAMICIDIN and TYROCIDINE)

Discovery

During the early thirties Dubos, at the Rockefeller Institute, was conducting studies on an enzyme which digested the capsular polysaccharide of *Pneumococcus*—Type 3. This enzyme was excreted by a spore-forming bacillus, isolated from soil. Its action was not restricted to hydrolysis of the purified polysaccharide *in vitro;* the enzyme was also able to digest the capsule as soon as it was produced by the living and multiplying pneumococcus. Not entirely satisfied with these results, Dubos wanted to go one step further. He considered the possibility that there also might exist bacterial enzymes capable of attacking not only soluble and isolated compounds but even the intact living cells of unrelated microbial species. With this idea in mind Dubos took soil samples and added to them, during the course of a whole year, suspensions of living pneumococci in the hope that, if such a pneumococcus-digesting organism was present in the soil, it would accumulate (14). However, Dubos never found such an enzyme, but, instead, isolated an organism from this soil mixture, which later was called "*Bacillus brevis*" (14, 15, 16, 17). Undoubtedly this organism had been present in that soil mixture in large amounts before the feeding experiment

was started. In all probability it did not "accumulate". *Bacillus brevis* does not feed on pneumococci, nor do pneumococci in any way incite excretion of a bactericidal substance by it; neither does it excrete an enzyme that digests living bacteria. Unintentionally Dubos had isolated a pure representative of the group of "bacterial antagonists", known since the time of Pasteur, and a study was undertaken in order to determine the properties of the bactericidal agents excreted by this antagonistic strain.

It was found that filtrates of a culture of *Bac. brevis* contain a substance which is highly bactericidal for a large group of Gram-positive bacteria, including pneumococci, streptococci and staphylococci. After much painstaking work, Dubos and his co-workers finally succeeded in isolating a crude material from cultures of it, which contained all the activity present in the original broth. This crude water-insoluble material, highly bacteriostatic for a large number of Gram-positive bacteria, was given the name "tyrothricin". Later it was separated into two crystalline fractions, gramicidin and tyrocidine (27, 18).

The same organism, as well as the two active principles, were isolated by the present author, independent of Dubos' work and at about the same time, during a study on inhibition of encapsulation of Friedlaender's bacterium by excretion products of soil bacteria (25, 26). It was observed that Friedlaender's bacterium, which under normal conditions produces enormous capsules of polysaccharides, failed to do this when grown in symbiosis with a soil bacillus which had been previously isolated from soil. Although no capsules were produced, the growth of Friedlaender's bacterium was not impaired by the presence of this soil bacillus. After an extensive study the active agent, excreted by this soil bacillus and responsible for this inhibition of encapsulation, was isolated, purified and crystallized. It was found to be identical with Dubos' tyrothricin (32).

Description of Bacillus brevis and the Conditions of Tyrothricin Formation

Colonies growing on nutrient agar: colonies circular, 1–2 mm. diameter after 24 hours incubation at 37° C.; flat, smooth, entire edges, white, pearl-like glistening, sometimes becoming slightly yellowish-brown on further incubation; mesophylic, strictly aerobic.

Morphology: actively motile, Gram-negative rods, single or in pairs; central spores; rods distinctly swollen during sporulation; endospores are ellipsoid.

Growth in nutrient broth: moderate clouding, slight pellicle as well as sediment.

Carbohydrates: no fermentation or acid production.

Litmus milk: after two days: reduction of litmus, milk clots, increasing peptonization, alkalinization.

Nitrate reduction: active reduction and nitrite formation.

Gelatin liquefaction: complete liquefaction of peptone-gelatin with considerable formation of ammonia.

Starch: no appreciable hydrolysis.

Indole: no formation in tryptophane broth.

Catalase: positive.

The foregoing description of the organism producing tyrothricin (14) is not in every respect identical with that of *Bac. brevis* in Bergey's "Manual of Descriptive Bacteriology", although the organism undoubtedly belongs in the large "brevis group". In the absence of criticism of taxonomists the organism has been generally accepted as *Bac. brevis*.

Relatively little is known of its physiology. A thorough systematic study on the conditions under which gramicidin and/or tyrocidine are produced has never been made. In shallow stationary cultures, one to two inches deep, tyrothricin is produced on a wide variety of media, such as prepared from peptones, tryptone, yeast extract, hydrolysed casein or gelatin, whey, potato extract or vegetable juices. Also, good yields are obtained with pure synthetic media containing glucose (1%) as the source of carbon, and one amino acid, such as asparagin (¼%), glycine or glutamic acid (½%) as the source of nitrogen, together with small amounts of inorganic salts, such as those of K, Mg, Na, Mn, Fe, Ca, $-PO_4$, $-SO_4$ or $-Cl$ (44).

Tyrothricin is elaborated already during the early logarithmic growth-phase and consequently cannot very well be a degradation product formed during autolysis. It accumulates during active growth of the organism and does not seem to be destroyed by further incubation. After one to two weeks there is usually no further noticeable increase in quantity. Yields of 300 mg. to 1 g. per liter of culture medium have been reported.

Growth of the bacterium involved and tyrothricin production do not always go together. Stokes and Woodward (44) showed that luxuriant growth without tyrothricin production occurs in aerated, submerged cultures of *Bac. brevis* on a large variety of complex nitrogenous media, in small tanks provided with stirring and aeration devices. However, when synthetic media of the composition given above were substituted, yields of 100 to 300 mg. per liter of culture medium were obtained by the submerged culture methods in tanks. No satisfactory explanation for this behavior can be given at present.

There are indications that tyrothricin produced by the submerged method contains a greater percentage of gramicidin than that produced by the stationary method. Tryptophane can not serve as a source of nitrogen in synthetic media for tyrothricin production. This is the more remarkable because this amino acid is one of the most important building components of gramicidin and tyrocidine.

In general it can be said that both gramicidin and tyrocidine are formed at the same time in a wide variety of media and under most divergent conditions. Tyrocidine usually is present in considerably larger amounts than gramicidin, about 4:1. No thorough study has ever been made to determine under what conditions production of tyrocidine is favored and under what conditions production of gramicidin is stimulated. One reason for this may be the fact that at present no suitable method is available to determine the concentration of both antibiotic agents separately in crude filtrates containing both.

Although gramicidin and tyrocidine are the main antibiotic fractions in the filtrates of *Bac. brevis,* there are undoubtedly other antibiotic fractions which have not been sufficiently investigated. Dubos, for instance, found a water-soluble fraction, behaving like a protein, and Referent has repeatedly found small amounts of an active fraction which is soluble in hot alcohol, but precipitates upon cooling. Both antibiotic fractions need further study.

Isolation of Bacillus brevis from Soil

A suspension of one part of ordinary garden soil in five parts of water is heated for ten minutes in a water-bath at 80° C. in order to destroy all non-sporulating species of bacteria. This pasteurized soil suspension is then streaked over the surface of several peptone

agar plates with a platinum wire. After 48 hours incubation at 37° C., the round flat and white colonies with entire edges and typical pearl-like glistening are isolated and inoculated into tubes with a 1% peptone–1% glucose–bromthymolblue–containing nutrient broth. When a number of different soil samples are used, little difficulty is encountered in finding separate colonies of the description given above. Tubes wherein no acid is produced and in which no pronounced pellicle growth is observed, usually are those of pure cultures of *Bac. brevis*. In order to prove this, one ml. of a three-day-old culture is added to 100 ml. of nutrient broth (without serum), inoculated with a Gram-positive organism such as *Pneumococcus* or *Streptococcus*. A dilution 1 : 100 of a three-day-old culture of *Bac. brevis* should exert enough bacteriostatic action to prevent growth of most of the Gram-positive species. Using this technique, Referent was able to isolate *Bac. brevis* from more than half of a large number of soil samples taken at random from several locations.

Preparation of Tyrothricin (Stationary Culture-Technique)

A very suitable and inexpensive medium for cultivation of *Bac. brevis* on laboratory scale is a 2% gelatin–½% casein solution (both commercial grades) in tap water. By addition of the required amount of trypsin, the proteins are digested by allowing the medium to stand for five hours at 37° C., adjusting the pH repeatedly to about 7.5 with NaOH. After digestion the medium is distributed in one-liter flasks or bottles in layers about two inches deep. Recently the use of press juice obtained from waste asparagus butts was advocated. This medium seems to give excellent yields of tyrothricin but is not readily available for laboratory-scale production at present. After sterilization each flask is inoculated with about 10 ml. of a 24-hour stock culture of *Bac. brevis* in the same medium. The flasks are incubated preferably at 35° C. for 7 to 14 days.

The content of the flasks is then harvested, the pH adjusted to 4.5 to 5 with concentrated HCl, and the heavy precipitate is allowed to settle overnight. The supernatant liquid is then syphoned off and discarded. To the remaining heavy suspension of tyrothricin and bacterial debris, methanol is added until a concentration of 60% is reached. This mixture is stirred frequently and allowed to

extract for 24 hours, preferably at somewhat elevated temperature. The supernatant liquid, rich in tyrothricin, is then syphoned off and the remaining debris extracted once more with 70% methanol. The debris is separated from the second extract by centrifugation.

The combined alcoholic extracts are filtered through paper, and an equal volume of 1% NaCl solution added.[2] A precipitate of tyrothricin is formed which is allowed to settle overnight in the refrigerator. The clear supernatant liquid is decanted and the precipitate collected on a Buchner funnel, washed twice with distilled water, and dried. When thoroughly dry, the powder obtained is extracted twice with ether for removal of inactive substances of lipoid nature. Tyrothricin is soluble in 95% alcohol up to at least a 5% solution. A considerable part of a dark pigment may be removed from such a solution by treatment with norite. The usual yield is one gram of tyrothricin per liter of culture medium.

Separation of Purified Tyrothricin into Gramicidin and Tyrocidine

Gramicidin is soluble in hot absolute acetone; tyrocidine is insoluble in acetone, provided the last traces of water have been removed and the tyrothricin itself is thoroughly dried over P_2O_5 in vacuum.

By extraction of dry tyrothricin with warm absolute acetone, a separation of both components can be achieved. To the acetonic solution of gramicidin is now added one volume of dry ether, and the precipitate is removed. The remaining solution is then evaporated under vacuum until a small volume remains. Crystalline gramicidin is obtained upon cooling. Further purification may be obtained by recrystallization from warm absolute acetone.

The absolute acetone–insoluble tyrocidine fraction is dissolved in about four times its weight of boiling absolute alcohol. A crystalline precipitate is obtained by addition of an alcoholic solution of HCl. The crystals of the HCl salt of tyrocidine may be recrystallized from absolute methanol.

Determination of Gramicidin and Tyrocidine in Mixtures and in Crude Filtrates

Optical rotation method: An alcoholic solution of pure gramicidin has an optical rotation of + 5, whereas tyrocidine under the same

[2] Instead of precipitation with 1% NaCl solution, the alcohol may be evaporated until only a water phase remains.

conditions gives an optical rotation of – 105. Tyrothricin or any other mixture of gramicidin and tyrocidine gives optical rotations between these extremes, and from the values observed the concentration of both components can be calculated. This method, of course, can be used only when no inert or other optically active impurities are present in the preparation.

Hemolysis method: The tyrocidine concentration found by the above method can be checked by another method based on the fact that tyrocidine is extremely hemolytic whereas gramicidin does not show hemolysis under the same conditions. An amount of 0.05 mg. tyrocidine added to 1 ml. of a suspension of fresh rabbit erythrocytes in 5% glucose solution causes complete hemolysis when incubated for two hours at 37° C. Even 0.005 mg. per liter causes partial hemolysis. Under the same conditions 0.5 mg. of gramicidin does not cause hemolysis.

This hemolytic property of tyrocidine has been utilized as a basis for a quantitative method of determining tyrothricin in culture filtrates of *Bac. brevis* (11). This, of course, is basically wrong, since, *a priori,* one may not assume that tyrocidine and gramicidin are excreted during the whole growth process in a constant ratio. Even different batches of tyrothricin, used for comparison, do not always contain the same ratio of tyrocidine and gramicidin. However, for the determination of tyrocidine, either in crude filtrates or in tyrothricin preparations, this method of Dimick would be well adapted when comparisons are made with pure tyrocidine instead of with tyrothricin. The method, in short, is based on measuring the degree of hemolysis of a standard suspension of rat erythrocytes under well defined conditions of medium, temperature and time of exposure. The degree of hemolysis, measured by photoelectric-colorimetric determination of light transmission, was found to be proportional to the amount of tyrothricin (tyrocidine?) added to the erythrocyte suspension. From the results obtained, a straight line calibration curve, covering the range of 4 to 10 micrograms per ml., was obtained. The amount of hemolysis produced by an unknown solution is measured and the tyrothricin (tyrocidine !) content is read directly from the calibration curve. For the assay of culture media, 1 ml. is added to 9 ml. of 95% alcohol. After thorough shaking the debris is removed by centrifugation and ½ ml. of the clear supernatant liquid is added to the standard erythro-

cyte suspension. For determination of the calibration curve the same broth and alcohol concentration is used. The accuracy is ± 5%. No satisfactory method for the determination of gramicidin, independent of tyrocidine, is known.

Chemistry of Gramicidin and Tyrocidine

Considerable work has been done to reveal the chemical structure of both gramicidin and tyrocidine. Although much progress has been made, insurmountable difficulties are still encountered, due to the nature of the compounds. Both gramicidin and tyrocidine are polypeptides of high molecular weight. The molecular weight of tyrocidine is 2,500 to 2,700; that of gramicidin is not definitely known but most likely is 1,500 or multiples of this value (51, 53, 46). By isothermal distillation methods a value of 3,100 was found; this value agrees well with that obtained by chemical analysis of crystalline flavianate (3,000), assuming one molecule of flavianic acid per molecule of gramicidin (53). However, cryoscopic determinations in camphor gave values of 1,340 to 1,622 (32); when cyclohexanol was used the values were as low as 600 to 1,200 (53). These low values indicate that gramicidin may dissociate reversibly under certain conditions into two or four units of lower molecular weight.

Both gramicidin and tyrocidine react positively in most of the common protein reactions, such as the biuret, xanthoproteic, Millon's and Voisenet reactions. On hydrolysis a mixture of amino acids is obtained, most of which have been identified (see Table 1). Gramicidin appears to possess the simpler structure. The following building stones, in the order of their importance, have been definitely identified: *l*-tryptophane, *d*-leucin, *d*- and *l*-valine, *l*-alanine, and a 1-amino-2-hydroxy compound which is possibly iso-serine. Glycin may also be present (47). According to Gordon (47), the number of amino acid building blocks per molecule of gramicidin in the arrangement given is 6:6:5:3:2(:2). These five or six amino acids are arranged either as a large cyclic molecule or as an end-substituted peptide chain.

The hydrolysate of tyrocidine contains the following amino acids: *l*-tryptophane; tyrosin; alanine; phenylalanine; aspartic acid; a basic amino acid, not yet identified; and ammonia.

According to Lipmann *et al.* (52), about 45% of the amino acids of gramicidin and 20% of those of tyrocidine are present in the

unnatural form. These observations have not been confirmed as yet under varied conditions of hydrolysis. If true it may form the basis for a satisfactory explanation for their antibiotic properties.

The most important chemical data of both gramicidin and tyrocidine are stated in Table 1.

Bacteriostatic and Bactericidal Properties of Gramicidin and Tyrocidine

Gramicidin undoubtedly is one of the most potent bacteriostatic agents known, equalled or only slightly surpassed in potency by pure penicillin. An amount of 0.01 to 0.02 mg. gramicidin per liter of broth is sufficient to inhibit the growth of most strains belonging to the species: *Diplococcus pneumoniae* (pneumococcus), *Strep. haemolyticus, Strep. faecalis, Strep. mastitides* and *Gaffkya tetragena* for a considerable period of time. Concentrations of 0.1 to 0.2 mg. gramicidin per liter of broth inhibits the growth of *Lactobacillus acidophilus, Staph. citreus* and *Mycobacterium phlei.* The most resistant Gram-positive species are *Staph. aureus, Bac. anthracis* and the sporulating anaerobes of the genus *Clostridium,* requiring 1 to 2 mg. of gramicidin per liter of broth. In order to prevent growth of all these species for an indefinite period of time, 10 to 20 times higher concentrations are required.

In general all species that are sensitive for gramicidin are also highly susceptible for tyrocidine. For the majority of susceptible species gramicidin is considerably more effective than tyrocidine. Usually five to ten times higher concentrations of tyrocidine are required to obtain the same inhibitory effect that gramicidin exerts. Interesting exceptions, however, are the spore-bearing species (*Bac. anthracis* as well as the gas-gangrene bacilli) where the ratio is almost the reverse. The same is true for meningococcus and gonococcus, two of the few Gram-negative species highly susceptible to both gramicidin and tyrocidine. Remarkably resistant to both gramicidin and tyrocidine are strains of *Staph. aureus.* Rammelkamp (38) observed that 12 of 19 recently isolated strains were permanently inhibited only by concentrations of tyrothricin ranging from 24 to 100 mg. per liter, and seven strains endured as much as 100 to 200 mg. per liter.

Undoubtedly the most outstanding property of gramicidin and tyrocidine, as well as of penicillin, is the almost perfect specificity

TABLE 1

	Gramicidin	Tyrocidine
Soluble in:	methanol, ethanol, dioxane, pyridin, glacial acetic acid and wet ethyl acetate, chloroform and acetone	
Insoluble in:	ether, petroleum-ether, benzole and carbon tetrachloride	
Chemical nature:	polypeptides, not digestible by proteolytic enzymes such as trypsin, papain, bacterial proteases, erepsin.	
Chemical analysis:	C: 62,7 % H: 7,6 % N: 14,8 % (Hoogerheide, 1940) (Hotchkiss-Dubos, 1941)	C: 59,6 % H: 6,7 % (– HCl-salt) N: 14,3 % Cl: 2,7 % (Hotchkiss-Dubos, 1941)
Molecular weight:	1340–1622 (in camphor) (Hoogerheide, 1940) 3000 (flavianate analysis) (Tishler *et al.*, 1941) 3100 (isothermal distillation) (Tishler *et al.*, 1941) 600–1200 (in cyclohexanol) (Tishler *et al.*, 1941) $(575)_n$ (Christiansen *et al.*, 1941) $(1500)_n$ (Hotchkiss, 1943)	2700 (Christiansen *et al.*, 1941) $(2500)_n$ (Hotchkiss, 1943)
Optical rotation [α_D]	+ 5°	– 105°

TABLE 1 (*Continued*)

	Gramicidin	Tyrocidine
Melting point:	228–232 (Hoogerheide, 1940) 228–230 (Hotchkiss-Dubos, 1940) 230–231 (Hotchkiss-Dubos, 1941)	230–234 (Hotchkiss-Dubos, 1940) 240 (Hotchkiss-Dubos, 1941)
Absorption spectrum:	Principle max. 2815 Å Secondary max. 2910 Å Minimum 2475 Å Extinction coeff. 12,6	
Positive protein react.:	Biuret, xanthoproteic (strong) Millon's (yellow color indicates tryptophane and no tyrosin) Voisenet (tryptophane)	
Negative protein reactions:	ninhydrin, Molisch	
Acid-base character:	neutral	weakly basic, with 2 amino groups and one carboxyl group
Amino acids accounted for:	*l*-tryptophane *d*-leucin *d, l*-valine *l*-alanine 1-amino–2-hydroxy-compound (iso-serine ?) (glycin)	*l*-tryptophane tyrosine alanine phenylalanine aspartic acid basic amino acid ammonia (dicarboxylic amino acid)

for Gram-positive bacteria. Whereas minute traces of these compounds inhibit the growth of practically all Gram-positive species, even large concentrations have no inhibitory effect whatsoever on the great majority of Gram-negative species. Although certain bactericidal dyes and detergents have tendencies in the same direction, none of the well known bacteriostatic or bactericidal chemicals such as phenols, mercurials, dyes or detergents exhibits a specificity for Gram-positive species in such extremes. A satisfactory explanation for this phenomena has never been given, but the close correlation between Gram-staining and tyrothricin activity is certainly not coincidental and is of importance enough to warrant a study of the problem. A few exceptions must be mentioned, however. We have already seen that a few Gram-negative species, such as gonococcus and meningococcus, are also susceptible to the action of both antibiotics. Moreover, several Gram-positive species, such as *Mycob. leprae, Mycob. ranae* and *Mycob. tuberculosum,* are only slightly susceptible to both gramicidin and tyrocidine.

The action of both components on Gram-positive bacteria is mainly bacteriostatic. However, higher concentrations, still relatively small, are decidedly bactericidal. When suspended in distilled water or a 5% glucose solution, a concentration as low as 0.1 mg. per liter of both agents will sterilize a pneumococcus suspension in 30 minutes at 37° C. For *Strep. haemolyticus* and *Corynebact. diphtheriae* (gravis), a concentration of 1 mg. per liter is required; for *Staph. aureus,* meningococci and gonococci 5 to 10 mg. per liter is necessary (42). In the absence of nutrient broth, using high concentrations of both antibiotics, several Gram-negative species, such as *Pasteurella avida, Past. tularensis, Shig. dysentheriae, E. coli, Salm. scottmuelleri* and *Eberth. typhosa,* are somewhat susceptible to the bactericidal action of gramicidin and especially tyrocidine. But even with tyrocidine, concentrations as high as 250 to 500 mg. per liter are necessary to obtain sterilization (13).

In the presence of broth, prepared from peptones, meat or yeast extracts, or meat infusion, the bactericidal activity of both antibiotics is greatly reduced. The Gram-negative species, with the exception of gonococci and meningococci, are not effected in the presence of broth, even not by tyrocidine. For Gram-positive species, about 10 to 50 times higher concentrations of both antibiotics

are required to obtain sterilization in broth. Addition of blood, serum, milk or other protein-rich substrates to the medium has a tremendous effect on the bacteriostatic and bactericidal efficiency of both compounds, especially on tyrocidine. Practically all bactericidal activity is lost in the presence of blood and serum, and both antibiotics remain only bacteriostatic (41). In order to obtain the same inhibition of growth of pneumococci in the presence of 10% serum, about 60 times as much tyrocidine and 10 to 20 times as much gramicidin is required than in the absence of serum. Small amounts of serum also greatly reduce the hemolytic activity of tyrocidine; 5% serum completely prevents hemolysis (31).

It has been observed repeatedly that bacteria, which are highly sensitive to tyrothricin, may develop marked resistance to this substance when subjected for a considerable time to increasing low concentrations of the bactericidal substance. The same increase in tolerance has been observed with strains of *Staph. aureus* isolated from an ulcer of the leg, treated for a considerable period of time with tyrothricin.

The specific action of tyrothricin on Gram-positive bacteria can be utilized to facilitate the isolation of certain tyrothricin-resistant Gram-negative species from mixtures with Gram-positive species. Isolation of *H. influenzae* from the nasopharynx is facilitated by the use of a medium containing sufficient tyrothricin to prevent the growth of Gram-positive cocci (42). Isolation of *Neisseria gonorrhoeae* is also facilitated by a concentration of 1/15,000 of tyrothricin in "chocolate" agar, preventing the growth of diphtheroids, streptococci and *Lactob. acidophilus* but not of *Staph. aureus* and gonococci. Numerous other applications in this field are possible and undoubtedly will be made in due time.

The effect of tyrothricin as a growth inhibitor is not restricted to bacteria; growth of higher plants is also strongly inhibited by small amounts. Growth of corn roots, when submerged in a tyrothricin solution containing 10 mg. per liter, is considerably retarded, and a concentration of 60 mg. brings growth practically to a standstill, permanently injuring the roots. The effect seems to be due to the tyrocidine fraction. This observation is of some importance, for it is possible, in view of the general occurrence of *Bac. brevis* in soil, that tyrothricin is produced locally in soils.

Mechanism of the Bactericidal Action of Gramicidin and Tyrocidine

The mechanisms of inhibition by gramicidin and tyrocidine are fundamentally different. Tyrocidine causes complete inhibition of all metabolic functions of the cell. The oxidation-reduction systems, respiratory-enzyme system and glycolytic enzyme system as well as dehydrogenase activity of *Staph. aureus* and *Strep. hemolyticus* are strongly inhibited by concentrations of 40 mg. per liter. Its action leads to disintegration of the cell and even lysis (19, 28). Some bacterial species are very easily lysed by tyrocidine; others, however, are not susceptible to lysis. A five-hour-old broth culture of *Micrococcus conglomeratus,* for instance, is completely lysed upon addition of 4mg. tyrocidine per liter (44). Gramicidin, on the other hand, only partially injures the cells. Cell metabolism may be either inhibited or stimulated, depending on the conditions, such as presence of K^+ ions or Na^+ ions. Synthesis of adenosine triphosphate is inhibited, and its bacteriostatic action may be due partially to such injury. Undoubtedly more research is required to give a clear picture of the mechanism of inhibition by both antibiotic substances.

Toxicity

Both gramicidin and tyrocidine are highly toxic for laboratory animals when injected intravenously, intraperitoneally or subcutaneously. However, given orally, both are non-toxic, even in large dose administered at once or over a long period. Apparently no absorption from the intestinal tract occurs and both agents are probably totally immobilized. Daily feeding of mice with 2 mg. of tyrothricin failed to cause any appreciable reduction of the *L. acidophilus* flora in the faeces, although this species is very susceptible *in vitro* to tyrothricin (45).

Tyrocidine, nothwithstanding its powerful hemolytic action, is considerably less toxic than gramicidin. The approximate lethal doses for 20-gram mice are:

intravenous:	tyrothricin	0.1 mg.
	tyrocidine	0.5 mg.
	gramicidin	0.075 mg.
intraperitoneal:	tyrothrycin	1.6 mg.
	tyrocidine	1.8 mg.
	gramicidin	0.6 mg.

Death is generally due to respiratory failure, occurring within 24 hours after injection of gramicidin or in three to four days with tyrocidine. Dogs, injected daily for ten days with small amounts of tyrothricin (0.2 mg. per kg.), showed only minor evidence of toxicity. However, a double dose caused death, usually before the ten injections were completed. Three-tenths of a milligram per kg. was not fatal but caused well marked acute or chronic changes in the liver, spleen, kidneys, heart and lungs.

Notwithstanding the high toxicity on parenteral administration, use of tyrothricin on open wounds has been found to be quite safe. Apparently very little absorption occurs. Even when injected into closed cavities, such as the bladder, the pleural cavity and the udder of cows, nc toxic effects are observed. For instance, a single injection of 200 mg. of tyrothricin into an infected pleural cavity of a human had no adverse effects (75).

Therapeutic Value

The extreme specificity of these compounds, exhibited against one type of living cell (Gram-positive bacteria) and not against another type (Gram-negative species), has drawn special attention to these compounds as possible therapeutic agents. Already Paul Ehrlich visualized that there might exist such specific chemical agents which would destroy pathogenic bacteria without a pronounced toxic effect on the cells of the host. However, besides this specificity, other factors also have to be taken in consideration. Briefly summarized, an ideal chemotherapeutic agent should possess the following properties:

a) high bactericidal or at least bacteriostatic properties for pathogenic bacteria;

b) body fluids, such as blood serum, exudate, should not inhibit this bactericidal action;

c) marked diffusibility in body fluids; therefore water solubility and of relative low molecular weight;

d) therapeutic ratio, the latitude between effectiveness against pathogenic bacteria and toxic effect on the host, as great as possible.

When we consider gramicidin and tyrocidine with these four criteria in view, it can readily be seen that both are remote from being ideal chemotherapeutic agents. Undoubtedly both are among

the most potent bacteriostatic agents known. However, as was pointed out already, serum and blood greatly reduce their bacteriostatic effectiveness. Both are practically water-insoluble; only colloidal solutions can be prepared; electrolytes cause complete precipitation; and diffusibility is extremely poor. Finally, the toxicity of both compounds is considerable.

It is evident, therefore, that both substances have been found of no value whatsoever for treatment of infectious diseases, such as pneumonia and generalized streptococcal and staphylococcal infections. There are numerous reports claiming that gramicidin is active *in vivo* and exhibits complete protection against many lethal doses of pneumococci, streptococci, staphylococci, *Bac. anthracis* and other pathogens susceptible to the action of this agent *in vitro*. These reports, although essentially correct, are misleading in so far as the evaluation of the therapeutic value of these compounds is concerned. Protection is afforded only when the infective dose and the protective dose are injected a short time apart and at the same site, *e.g.,* both intraperitoneally, thus giving a direct contact of the germicide with the infective organisms before the latter have had an opportunity to invade their host.

As soon as infective and protective injections are given on different sites and the bloodstream has to transport the antibiotic agent to the site of infection, both gramicidin and tyrocidine are entirely ineffective, even in doses approaching the sublethal range.

However, for topical application and local use, as in the treatment of infected wounds, especially when not much exudate is present, both gramicidin and tyrocidine have proved to be of great value. One of the first practical applications of tyrothricin was in the field of veterinary medicine. Suspensions of it are used quite extensively for local treatment of bovine mastitis, a common disease in dairy cattle, usually caused by *Strep. agalactiae*. This organism is resistant to most of the sulfa drugs but very sensitive to tyrothricin. Considerable literature has accumulated on the treatment of this disease with tyrothricin, and there seems to be general agreement that it is one of the least irritating agents of several chemotherapeutic agents commonly used for treatment of this disease. Moreover, tyrothricin has been shown to give the highest percentage of recoveries, compared with other drugs. Often 80% to 90% of all cases treated are cured, usually with one treatment especially if the

treatment is given when the quarters are dry (non-lactating cows). The solutions of tyrothricin, either in distilled water or as a water-mineral oil emulsion, are instilled into the udder *via* the teat-canal and left there for 12 to 18 hours. About 25 ml., containing from 30 to 150 mg. tyrothricin per quarter, gave the best results.

On humans tyrothricin is used very successfully for the treatment of infected ulcers, abscesses, sinusitis, osteomyelitis, empyema and suppurative wounds. It should be borne in mind, however, that good surgery should always be combined with application of tyrothricin, since this agent must be able to come in direct contact with the infecting organisms; one cannot depend on possible diffusion through tissue, exudate, pus or debris. Tyrothricin is most effective in dealing with streptococcal infections and considerably less effective in staphylococcal infections. This, of course, could be expected from *in vitro* results. For wound disinfection tyrothricin solutions are very satisfactory and cause no irritation or toxic effects. It is surprising that its use for this purpose has not been more extensive, especially for prevention of infection, including war wounds. Tyrothricin apparently is just as satisfactory for this purpose as gramicidin, and for therapeutic use there is no need to separate tyrothricin into its two fractions. The concentrations used for wound treatment vary considerably with the investigator. For large wounds as well as for treatment of empyema, 100 mg. of tyrothricin per liter of distilled water are sufficient. For smaller areas, however, higher concentrations can be used safely. For instance, for treatment of ulcers concentrations as high as five grams per liter have been used. Since it is impossible to prepare stable colloidal solutions of such high concentrations in water, alcoholic solutions or tinctures must be used. Solutions for wound treatment are either used in the form of wet dressings or directly sprayed on the wounds.

ANTIBIOTIC AGENTS EXCRETED BY ACTINOMYCES SPECIES

Considerable work has been done on the isolation of antibiotic agents excreted by species of *Actinomyces*. The name of Dr. S. A. Waksman is intimately connected with practically all research done on this type of antibiotics. This is hardly surprising, since this investigator is generally recognized as the most outstanding expert on this particular genus of micro-organisms. A large number of

antagonistic Actinomycetes have been isolated by him and his co-workers, and a number of strains have been investigated more thoroughly. Some of the antibiotic agents have been isolated in chemically pure form; others have been highly purified. The most important are actinomycin A, actinomycin B, actinomycetin and streptothricin.

Actinomycin A and B

Waksman and Woodruff (83) isolated from soil in 1940 a new chromogenic species of *Actinomyces* which showed strong antagonistic properties toward all bacteria belonging to both the Gram-positive and Gram-negative types. This new species was later described as *Act. antibioticus.*

The antibiotic agent responsible for this inhibition is excreted on a large variety of media, varying from media rich in protein or peptones to pure synthetic media with starch as the source of carbon and a nitrate or, better, phenylalanine as the only source of nitrogen. Small amounts of agar are added to these media in order to prevent the delicate surface membrane from being submerged. The active agent can be extracted with ether from six- to eight-day-old cultures of this organism on nutrient media. Upon evaporation of the ether extract a crude powder is obtained which was called "actinomycin". By means of petroleum-ether, actinomycin can be separated into two crystalline fractions. Actinomycin A is insoluble in petroleum ether but limitedly soluble in water, and is highly pigmented (red). Actinomycin B, on the other hand, is soluble in petroleum ether but not in water, and is not pigmented. Fraction B is formed mainly during the early stages of growth, whereas fraction A is produced principally in older cultures.

Actinomycin A is the more active fraction and has been studied most extensively. One-one-hundredth of a microgram per ml. of broth is sufficient to inhibit the growth of several Gram-positive species, such as streptococci, staphylococci and *Bac. subtilis.* This activity is of the same order as that of gramicidin and penicillin. Other Gram-positive species, however, require as much as 10 micrograms per ml. (*Mycobact. tuberculosum, Bac. macerans*). Gram-negative species are also inhibited, although usually concentrations of 10 to 20 micrograms per ml. are required, even 100 micrograms for the coli-aerogenes group and *Serratia marcescens.* Generally speaking, Gram-negative species are more resistant to actinomycin

A than Gram-positive forms, but the sharp demarcation line between the two groups in regard to their sensitivity to gramicidin or penicillin does not exist for actinomycin A. There are marked differences in sensitivity for this antibiotic within the species of each group.

The extreme inhibitory action of actinomycin A is mainly of a bacteriostatic nature, similar to the action of gramicidin. Like the latter, only when much higher concentrations are employed, does bactericidal action become evident; *e.g.,* 10 to 50 micrograms per ml. are required for certain sensitive Gram-positive species. Actinomycin B, on the other hand, lacks the bacteriostatic range which precedes bactericidal action. Its effect is mainly bactericidal, and in this regard it resembles tyrocidin. The bactericidal potency of actinomycin A and B is of the same order.

Actinomycin is also quite fungicidal, especially against fungi of the genus *Penicillium.* It is also quite active against several actinomycetes.

Actinomycin A is a crystalline substance of a melting point of 250° C. and is optically active $[\alpha_D^{25}] = -310 \pm 5$. It has a molecular weight of about 800 and the following composition: C—59.0%; H—6.8%; N—13.4%; O—20.8%. It is a polycyclic N-compound, the exact configuration not being known. Several groups can be acetylated, and it is a reversible oxidation-reduction system, apparently of the quinone type. In this regard it resembles chlororaphin, the antibiotic pigment of *Pseudomonas chlororaphis.* Actinomycin A is readily soluble in chloroform, benzene and ethanol, moderately soluble in acetone and hot ethyl acetate, and sparingly soluble in water and ether. It is quite stable; a neutral solution can be boiled for 30 minutes without loss of activity; however, in acid or alkaline solution it is slowly decomposed. From among 250 strains of actinomycetes tested it was found to occur only in strains of *Act. antibioticus.*

Since actinomycin A is sufficiently soluble in water, highly bacteriostatic and not inhibited in its action by the presence of serum or other body fluids, it has most of the properties required of a good chemotherapeutic agent. Unfortunately, however, it is extremely toxic, even in oral administration. Its L.D.-50 (dose lethal for 50% of the animals tested) is as low as 0.35 mg. per kg. for mice, compared with 30 mg. for gramicidin and 90 mg. for tyrocidine.

Death apparently is due to respiratory failure and usually does not occur until 15 to 20 hours after administration. Even such traces as 50 micrograms per kg., given intraperitoneally, produce death in mice or rats when administered daily for six days. Gross pathological changes can be observed, such as marked shrinkage of the spleen. Liver and kidney functions are also affected. Actinomycin A is rapidly removed from the blood, partly excreted in the urine and partly stored in many organs of the body.

Due to its extreme toxicity, actinomycin A has no therapeutic possibilities, not even for topical application on open wounds.

Active actinomycin-containing filtrates can be produced by growing *Act. antibioticus* in flasks in shallow layers of tryptone-glucose broth. The composition of the medium as well as the technique for obtaining good surface growth will be outlined in more detail in the discussion of streptothricin. Actinomycin filtrates of considerably higher titer can be produced by the submerged culture technique. This technique is becoming increasingly important in research on as well as production of antibiotics, especially penicillin, streptothricin, actinomycin and tyrothricin. It consists of growing strictly aerobic species of bacteria, actinomycetes or fungi under submerged conditions and supplying the culture with a generous amount of sterile air, either by continuous vigorous agitation in flasks or by passing an uninterrupted stream of sterile air through the nutrient liquid. Often it is necessary to combine this aeration with vigorous stirring to disperse the air bubbles. Growth of actinomycetes and fungi under such conditions is entirely different from the pellicle or matt observed in stationary cultures. Discrete colonies and mycelial fragments are produced throughout the liquid. Growth is much more rapid and abundant and of surprising uniformity. Consequently results obtained by the submerged culture technique are more reproducible, and usually higher titers of the antibiotics are obtained in a shorter period of time. Moreover, this technique is ideally adaptable to large-scale production of antibiotics in tanks equipped with aeration devices and provided with mechanical stirrers. For laboratory research, 500-ml. Erlenmeyer flasks with 150 to 200 ml. of inoculated media, continuously shaken by a shaking-machine in a constant temperature room, are very well adapted for submerged culture study of actinomycetes and fungi. The importance of this newly developed technique for the study of antibiotics can not be stressed too much.

As far as actinomycin production is concerned, filtrates may be obtained after an incubation period of one week, inhibiting the growth of *Bac. subtilis* in a dilution of 1/200 for stationary cultures or 1/400 for submerged cultures. Actinomycin A is not produced until maximum growth has been reached, usually not before the fifth day. It is, therefore, entirely possible that it is an autolytic product, liberated after active growth has ceased.

Actinomycetin

Strictly interpreted, actinomycetin is not an antibiotic agent. However, it is usually discussed with antibiotics and therefore will be considered here briefly. In 1936 it was observed by Welsch that during sporulation a large number of *Actinomyces*-strains (about 50% of all strains tested) liberate a bacteriolytic agent into the medium. This agent causes lysis of a large variety of heat-killed Gram-positive and Gram-negative species. This bacteriolytic principle (actinomycetin) is produced only in older cultures of *Actinomyces* on a large variety of media supporting good growth of these organisms. Broth of neutral or slightly alkaline pH distributed over flasks in shallow layers, gives the highest yields. The lytic action of crude actinomycetin is especially pronounced on suspensions of heat-killed Gram-negative bacteria. Clarification is achieved in a few hours. Heat-killed Gram-positive bacteria are also lysed, but at a much slower rate; usually 24 to 48 hours incubation is required.

Living streptococci and pneumococci may also be lysed, but only by filtrates of a restricted number of *Actinomyces* strains. There are indications that these strains excrete weakly bacteriostatic or bactericidal agents which may destroy the living cells before they become susceptible to the lytic action of actinomycetin. For instance, by extraction with ether of certain crude actinomycetin preparations, Welsch isolated a fraction from the ether phase which inhibited the growth of *Staph. aureus,* pneumococci, streptococci and several species of the genus *Bacillus* in concentrations of 0.2 mg. per ml. However, very little attention has been paid to these bactericidal fractions, and it is entirely possible that the combined action of such bactericidal impurities with actinomycetin is the reason for the lysis of living bacteria.

Actinomycetin (the bacteriolytic fraction) is a protein, soluble in water, glycerol and dilute saline, and is precipitated by alcohol,

acetone or ammonium sulphate. It is heat labile, being inactivated by exposure to temperatures of 60° to 70° C. It is also inactivated by ultra-violet light of a wave length shorter than 300 mμ.

Streptothricin

The name "streptothricin" is derived from the early generic designation, *Streptothrix,* given to the group of organisms now known as *Actinomyces.* It is one of the latest additions to the growing list of antibiotics, and there are indications that this substance may become an important chemotherapeutic agent. There is relatively little information available about it, but several laboratories are doing considerable research on the substance.

Streptothricin was first isolated as a crude extract by Waksman and Woodruff (87) from culture filtrates of a strain of *Actinomyces* isolated from soil and later identified as *Act. lavendulae.* It was found that different strains of this species vary widely in their ability to produce streptothricin. It is essential, therefore, that a very active strain be used for streptothricin production.

Production of active streptothricin filtrates. The following medium gives good yields of streptothricin (88, 89):

1% starch (or glucose)
0.5% tryptone
0.05–0.2% K_2HPO_4
0.05–0.2% NaCl
0.01% $FeSO_4$
} per liter of tap water.

Amounts of 200 ml. or less (pH may vary between 5.0 and 7.5) are distributed over one-liter Erlenmeyer flasks and sterilized for 15 minutes at 10 lbs. pressure (glucose should be sterilized separately). A shallow layer of medium, not more than one inch thick, is essential for good production; the shallower the layer the higher the ultimate titer. Each flask is inoculated with spores of *Act. lavendulae* from a slant or agar plate, care being taken that the spores remain afloat on the surface of the medium. This can usually be achieved by mixing the spores with sterile talcum powder and using about 100 mg. of the mixture per flask. The spores on the surface develop into a dry entire pellicle which should not be disturbed. Spores that become submerged develop into submerged colonies which do not produce streptothricin. In order to prevent the pellicle from falling to the bottom, ¼% agar may be added to the me-

dium, or absorbent cotton packed up to the surface of the liquid may be used. When starch is employed in the medium and talcum powder on the surface, further precautions are usually unnecessary. Good streptothricin production depends largely on good growth and good growth is correlated with maintenance of a dry pellicle.

The inoculated flasks are incubated for 10 to 14 days at 20° to 25° C. At higher temperatures (maximum 30° C.) streptothricin is produced in a shorter time but the ultimate titer is considerably lower. There is a definite optimum incubation period; beyond this the streptothricin yields decrease rapidly. A dilution of 1/200 to 1/500 of a good active filtrate of *Act. lavendulae* is capable of inhibiting the growth of *Bac. subtilus,* and a dilution of 1/50 to 1/100 will prevent growth of *E. coli.*

Glutamic acid or glycin may be substituted for the tryptone in the medium with comparable results.

A second method of producing active filtrates of *Act. lavendulae* is by the submerged culture technique, either in flasks shaken continuously by mechanical means or in vessels provided with stirring devices, by passing sterile air through the medium. Both procedures lead to abundant submerged growth in a very much shorter time period than under corresponding stationary surface conditions. Streptothricin production under submerged conditions is not subjected to such extreme variations as sometimes are encountered in surface cultures; moreover, considerably higher titers are obtained. Maximum activity is usually reached in five to seven days and coincides with maximum growth. Substitution of 3% neutralized corn steep liquor or 0.6% soybean meal for tryptone in the medium increases the titer considerably.

Isolation of streptothricin. The active filtrates are collected, filtered, and shaken with Norite A. The charcoal which absorbs all activity from the filtrate is collected on a filter and extracted with acidified alcohol. After neutralization of the extract with NaOH, ten volumes of ether are added. A highly concentrated aqueous solution of streptothricin separates. It may be concentrated further by evaporation at reduced pressure. Although streptothricin has not been crystallized, highly purified preparations have been prepared, but not much information on the technique for purification is available. Streptothricin is a nitrogen-containing base, insoluble in ether, petrolether and chloroform, but soluble in water.

Characteristics of Actinomyces lavendulae. During growth in complex nitrogenous media, both as shake culture and as stationary culture, there is rapid utilization of organic nitrogen compounds, far in excess of the requirements for cell synthesis. Considerable deamination occurs and ammonia is liberated. Carbohydrates are also vigorously attacked and acids are produced, largely lactic acid. During the early stages of growth, acid production predominates over deamination. Consequently, a drop in pH is observed from 7.0 to about 6.0, followed by a steady rise during the later stages of growth. Maximum streptothricin titers are reached as soon as this rise in pH becomes apparent. The drop in pH can and should be largely controlled by choosing the proper ratio of carbohydrate and protein. When this ratio is too great, a sharp drop in pH is observed, sometimes as low as 3.2; when it is too low, the medium becomes very alkaline, due to accumulation of ammonia.

Characteristics of streptothricin. Streptothricin is unique as far as its bacteriostatic specificity is concerned. Gram-negative and Gram-positive species are equally susceptible to its action. However, in both groups there are species that are highly resistant, and others that are highly susceptible. For instance, among the Gram-positive species, *Bac. subtilis* is very sensitive, but a genetically closely related species, *Bac. mycoides,* is resistant. Among the Gram-negative species, *E. coli, Brucella abortus* and several species of *Shigella* are very sensitive, but *Ps. fluorescens* is resistant.

Streptothricin is bactericidal as well as bacteriostatic. It is quite soluble in water, relatively stable and heat-resistant; it is active in the presence of serum and has low toxicity for animals. These properties, of course, are of particular importance for possible therapeutic application. However, only *in vivo* experiments of a preliminary nature have been reported, and its therapeutic possibilities have not been fully explored.

ANTIBIOTIC SUBSTANCES FROM OTHER SOIL BACTERIA

It has been pointed out already that there is an unlimited number of bacteria in the soil that can excrete bactericidal substances under proper conditions. An extensive field for valuable research is here awaiting exploration, and it is possible that valuable chemotherapeutic agents may be discovered. Unfortunately, little attention has been paid to it, and the promising results reported in the litera-

ture have never been carried further. Although a large number of antibiotic substances derived from soil bacteria have been discovered, only very few of them have been isolated, purified, identified and tested for possible therapeutic value. The most important are pyocyanase and pyocyanin.

Both these substances are excretion products of certain strains of *Pseudomonas aeruginosa.* Undoubtedly this was the first important antagonist to receive attention. In general, excretion of antibiotic substances by soil bacteria as well as by molds is highly "strain-specific" and not "species-specific". Often a considerable decrease or even loss of the ability to excrete such agents may be observed on subculturing. This is especially true of *Ps. aeruginosa.* Some strains produce only pyocyanase, others produce both pyocyanase and pyocyanin, whereas still others produce no active agent at all.

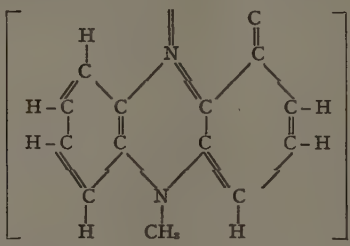
According to Goreczky (108), excretion of pyocyanase is closely paralleled by the virulence of the strain. It is produced only on complex nitrogenous media and not in synthetic media. Glycerol-bouillon apparently gives optimal yields. It may be prepared by extraction of a culture medium of a suitable strain with ether or petroleum-ether. This crude extract consists mainly of a neutral fat, a phosphatide, both inactive, and a mixture of fatty acids of high molecular weight possessing hemolytic, bacteriostatic and weakly bactericidal properties. The unsaturated fatty acids are the most active, their activities being proportional to their number of double bonds.

Pyocyanase is active against a large variety of Gram-positive as well as Gram-negative species including *Bac. anthracis, Corynebacterium diphtheriae, Strep. haemolyticus, Staph. aureus* and *albus, E. coli, Eberth. typhosus, B. dysenteriae* and *Brucella abortus.* It is highly haemolytic, however, as well as surface active. Its toxicity for animals is relatively low. For a method of preparation in reasonably pure form, see 113 and 116 in the bibliography.

Pyocyanin is a blue-green pigment elaborated by many strains of *Ps. aeruginosa* on complex nitrogenous media as well as on pure synthetic media with NH_4-lactate as the source of nitrogen and carbon. It can be obtained in good yield by growing the organism for 10 to 14 days at 25° C. in very shallow layers of a medium containing 1% peptone, ½% NaCl and 12% gelatin. It may be ex-

tracted from the slightly alkaline culture media with chloroform in which it is soluble with a dark blue color. From the chloroform phase it can be taken up in small amounts of dilute HCl in which it is soluble with a deep red color. After addition of bicarbonate it can be taken up again in chloroform, and after thorough drying of the chloroform solution with water-free Na_2SO_4, it is precipitated as the crystalline HCl-salt by exposing the chloroform solution to HCl vapors.

Pyocyanin is a methyl derivative of α-hydroxy-phenazine. It has been synthesized by Wrede from this compound by treatment with dimethylsulphate (125–129). Its structure is:



Pyocyanin inhibits the growth of numerous species of bacteria, Gram-positive as well as Gram-negative, yeasts and fungi. Its activity in general exceeds that of pyocyanase; it is not excessively toxic; and in small concentrations it markedly enhances the respiration of many bacteria species as well as tissue cells.

Other bacterial pigments belonging to the phenazine-group. The yellowish-green pigment produced by *Pseudomonas chlororaphis* (chlororaphine) as well as that produced by *Chromobacterium iodinum* are related to pyocyanin and have been chemically identified. Chlororaphine is a quinhydrone-like complex of equimolar proportions of phenazine-*l*-carboxamide and dihydrophenazine-*l*-carboxamide; the pigment produced by *Chrom. iodinum* is probably NN'-dioxide of 1,2-dihydroxy phenazine. The latter pigment is quite active against *Strep. haemolyticus* and other Gram-positive organisms. An amount of 2 mg. per liter prevents growth of these organisms. This concentration is of the same order as for pyocyanin but about a hundred times as much as required for the more potent antibiotics, gramicidin, actinomycin or gliotoxin.

In general, it is very difficult to compare the activity of different antibiotics investigated by different investigators from the data available in the literature. It would be highly advisable, therefore, that a comparative study be made of the bacteriostatic and bactericidal properties of pure pyocyanase, pyocyanin, chlororaphin, the pigment of *Chrom. iodinum* and of many other antibiotics, under strictly comparable conditions against a number of the common pathogens in the presence and absence of serum. The 50% lethal dose and the possible therapeutic values of these compounds should also be determined.

Antibiotic agent from Pseudomonas fluorescens. Lewis (134) observed that a strain of *Ps. fluorescens* produces a thermostable, dialyzable agent, bacteriostatic and bactericidal for a large number of bacteria species, especially those of the genera *Bacillus* and *Micrococcus.* Representatives of the coli group, however, were resistant. The active principle is soluble in alcohol and adsorbed by charcoal, but has not been studied further.

Antibiotic agent from Serratia marcescens. Eisler and Jacobsohn (131) found that sterile broth cultures of a particular strain of *Serratia marcescens* contained a thermostable substance which inhibited the growth of *Corynebacterium diphtheriae,* gonococci and other species. No further work has been done with the active principle.

Antibiotic agents from representatives of the genus Bacillus. Undoubtedly the highest percentage of antagonistic strains of soil bacteria is found in the genus *Bacillus.* The excretion products of *Bac. brevis* are the only antibiotics of this group that have been investigated thoroughly. However, many related species excrete similar antibiotics which are not identical with gramicidin or tyrocidine. The fact that antibiotic products can be isolated by using the same procedure, as for tyrothricin, made certain investigators conclude that the active agent is tyrothricin. Usually such a conclusion is not permissible, even when the "bacterial spectrum" is similar to that of tyrothricin. Referent found on at least two occasions that the absorption spectrum in ultra-violet light of two such agents, isolated from two unidentified *Bacillus*-strains, was entirely different from that of gramicidin and tyrocidine, proving that both are chemically different compounds.

As early as 1940 Referent pointed out that certain strains of the most common spore-formers, among which was *Bac. subtilus,* ex-

crete bactericidal products during growth in broth. Small amounts of crude active material were isolated by methods similar to those described for tyrothricin, but none of these crude active fractions was further investigated. Katznelson (1942) and Himfeld and Feustel (1943) confirmed the excretion of antibiotic substances by strains of *Bac. subtilis.* The active filtrates were found to be heat stable and effective against *Micrococcus conglomeratus* and *Staph. aureus.* The active principle has not been isolated and purified as yet, and nothing is known of its activity *in vivo.*

Water-soluble, highly active, bactericidal agents were also found in cultures of *Bac. vulgatus,* and an unidentified yellow spore-bearing bacillus (130). These agents were effective against various bacteria and fungi, including *Staph. aureus, Strep. lactis, E. coli, Eberth. typhosus* and *Lactob. acidophilus.*

Stokes and Woodward (135) isolated highly potent extracts from several antibiotic soil organisms, including *Bac. mesentericus* and *Bac. adhaerens,* which were active against various Gram-positive bacteria, but no further work has since been published on these agents.

Antibiotic substances against plant pathogens. An extensive literature has accumulated on this subject, indicating that antibiotic substances excreted by certain species of soil bacteria may be active in the natural control of soil-borne plant diseases of bacterial or fungal origin. None of such antibiotic substances has as yet been isolated chemically pure or even as a crude extract. Literature on strains of molds and soil bacteria, antagonistic to plant pathogens such as different species of *Fusarium, Ustilago zeae, Helminthosporium sativum, Ophiobolus graminis* and *Phymatotrichum omnivorum,* has been extensively reviewed (145). A great variety of antagonistic strains of molds and soil bacteria, many of them unidentified, have been isolated and found to excrete antibiotic substances against plant pathogens. Certain strains of *Bac. simplex* and *Bac. mesentericus* and some species of *Pseudomonas* and *Achromobacter* are a few examples.

Seeding of soil with such antagonistic strains or spraying with culture filtrates of them was found in some cases to check the prevalence of certain plant pathogens and to prevent disease. No systematic study of this subject has been made. The practical significance of prevention or cure of plant diseases by use of antibiotic sub-

stances should not be underestimated, and a thorough study of this subject seems more than justified.

ISOLATION OF ANTAGONISTIC BACTERIA SPECIES FROM SOIL

The following procedure has been used by Referent very successfully for the isolation of a large number of soil bacteria, excreting antibiotic substances.

A sterile 1% peptone-agar medium is inoculated just before solidification with a few drops of a suspension of *Mycobact. phlei* in sterile distilled water. This organism is quite susceptible to antibiotic substances and is slow growing. Any other organism that possesses a combination of these two properties can also be used. After pouring a number of plates with this inoculated agar, each plate is streaked with one drop of a dilute soil suspension. The dilution of the soil suspension should be such that a large number of separate colonies is produced without too much crowding. A few drops of glycerin on a small piece of filter paper placed in the cover of the Petri dish, will avoid "spreading" of certain types of colonies. The plates are incubated at 30° to 35° C. for two days. Several colonies on the agar surface may then be found surrounded by a more or less pronounced sterile halo, indicating excretion of bactericidal substances against *Mycobact. phlei.* Colonies surrounded by a sterile halo are isolated and usually prove to be equally active against a large variety of other species. The principle of this technique is to suppress the growth of the test organism (*Mycobact. phlei*) sufficiently long in order to allow time for the "antagonist" to develop into a colony and excrete sufficient antibiotic substances before the test organism develops.

A second method successfully used by Referent for obtaining antagonistic strains was similar to that just described, but sterile agar was used. As soon as the colonies of the soil bacteria had developed (usually after 24 hours incubation) the remaining spaces between the colonies were inoculated with *Bac. mycoides.* This organism forms colonies that gradually cover the total surface of the plate. However, it avoids those colonies that have excreted bactericidal substances.

The great majority of antagonistic species isolated by both methods belonged to the genus *Bacillus.* However, also antagonistic strains of *Pseudomonas* and *Actinomyces* were obtained.

If representatives of the genus *Bacillus* are desired it is better to pasteurize the soil suspension before streaking it on nutrient agar. For isolation of *Pseudomonas* antagonists, surface water is used instead of soil suspensions, and for *Actinomyces* antagonists, compost suspensions or other material naturally rich in such species.

As has been pointed out before, a whole field for research is wide open here. The simple technique for isolation of antagonists given above makes an abundance of antagonistic strains available for study to every bacteriologist interested in the subject. Although the soil microbiologist has a professional advantage and is more familiar with bacterial soil population than the average bacteriologist, there is no need for the latter to wait until supplied with antagonistic strains. However, collaboration with a chemist is often advisable, especially for isolation, purification and identification of the active agents. Isolation and description of antagonistic strains and evaluation of crude filtrates is of little value, since the involved data often are available in the bacteriological literature of half a century ago. Attempts should always be made to isolate, purify and identify the active agent. Its "bacterial spectrum" should be determined in the presence and absence of serum, its toxicity for laboratory animals, and finally its therapeutic value in experimental infections, avoiding direct contact between the infective organism and the therapeutic agent. Investigations of "crude extracts" should be limited, especially when it is possible to obtain purer products by further study. Often those crude fractions are mixtures of two antibiotics and give confusing and misleading results.

Finally, a few words on the nomenclature of new antibiotics. The most logical designation undoubtedly is a name related to that of the species from which an antibiotic is obtained and not one related to the genus. Names such as penicillin and actinomycin may become misleading as soon as similar bactericidal agents are isolated from other species of the same genera.

SUMMARY AND CONCLUSIONS

The isolation of *Bacillus brevis* by Dubos in 1939 and the subsequent study of the active principles excreted by this species, have opened a new field for discovery of new chemotherapeutic agents for human, animal and possibly also for plant diseases.

Gramicidin and Tyrocidine. Both these agents have been isolated from cultures of *Bac. brevis.* This organism is a common inhabitant of the soil and may be isolated by a special technique. Both agents are polypeptides and have been obtained in crystalline form, chemically pure. Although the amino acid building components are known for both substances, their chemical configurations can not be determined with our present knowledge of protein chemistry. Both agents act almost exclusively on Gram-positive bacteria, especially on pneumococci and streptococci. This extreme specificity is quite unique. Moreover, both agents represent the most potent bacteriostatic substances known. Serum inhibits their action greatly. Since both are quite toxic, they are not suitable for internal use as chemotherapeutic agents. For local treatment of infected wounds, ulcers, *etc.* they have been found to be of great value.

Actinomycin A and B. Both these substances are isolated from cultures of *Actinomyces antibioticus* and have been prepared in crystalline form. Actinomycin A, a red pigment, is the more active, comparable in activity with gramicidin. It acts mainly on Gram-positive species but lacks the extreme specificity of gramicidin. Higher concentrations of actinomycin A also inhibit Gram-negative species. This substance is a polycyclic N-compound, but the exact configuration is not known. Its extreme toxicity, even by oral administration, makes it unsuitable for therapeutic use. Actinomycin B is considerably less active and has not been studied very extensively.

Actinomycetin. This agent is excreted by a large number of *Actinomyces* species. It is a lytic agent and, strictly speaking, not an antibiotic agent, since for its lytic action mainly dead bacteria are necessary. Often, however, it is contaminated with unknown antibiotic agents.

Streptothricin. This agent is an excretion product of *Actinomyces lavendulae.* It does not exhibit specificity for Gram-positive species, but some of the latter as well as certain Gram-negative species are quite sensitive to it. It is water-soluble, active in the presence of serum and has low toxicity for animals. It may become an important chemotherapeutic agent.

Other antibiotic agents. Among the antibiotic agents, known long before interest in this field was wide-spread, are:

a) pyocyanin, the blue-green pigment of *Pseudomonas aeruginosa;* this substance is less active than either streptothricin, actinomycin or tyrothricin, but shows activity against a large variety of Gram-positive as well as Gram-negative species; it has been crystallized, its structure is known and it has been synthesized;

b) chlororaphin, the pigment of *Pseudomonas chlororaphis,* chemically related to pyocyanin but is less active;

c) a number isolated from cultures of *Pseudomonas fluorescens, Serratia marcescens, Bacillus subtilus* and other spore-formers; they have not been purified and studied sufficiently.

It is not so much the few antibiotic agents that have been isolated, partly or completely identified and studied, that comprise the main contribution of soil microbiology to chemotherapy. In fact, it is entirely possible that very few of the many agents excreted by antagonistic soil bacteria will prove to be of any value chemotherapeutically. The important feature is that after an antibiotic agent has been identified chemically, it becomes possible to prepare derivatives of it that may be far better than the mother substance produced by micro-organisms. In other words, *via* antagonistic soil bacteria and molds and isolation and identification of the antibiotic agents excreted by such antagonistic strains, the chemist is guided to a class of compounds among which valuable chemotherapeutic compounds may be hidden. Soil microbiology may even become the basis on which chemotherapy can develop from the almost complete empirical stage of today to a broad science of utmost importance tomorrow. Although very little attention so far has been paid to antibiotic substances against plant pathogens, including fungi, the broad principles outlined in this review are also adaptable for this group of pathogenic organisms. A valuable research field is waiting here for proper exploration.

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